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Endogenous modulation of TrkB signaling by treadmill exercise after peripheral nerve injury

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Abstract

After peripheral nerve injury, transected fibers distal to the lesion are disconnected from the neuronal body. This results in target denervation but also massive stripping of the central synapses of axotomized motoneurons, disrupting spinal circuits. Even when axonal regeneration is successful, the non-specific target reinnervation and the limited rebuilding of spinal circuits impair functional recovery. Therefore, strategies aimed to preserve spinal circuits after nerve lesions may improve the functional outcome. Activity-dependent therapy in the form of early treadmill running reduces synaptic stripping, mainly of excitatory synapses, and the disorganization of perineuronal nets on axotomized motoneurons. The mechanism underlying these effects remains unknown, although the benefits of exercise are often attributed to an increase in the neurotrophin BDNF. In this study, TrkB agonist and antagonist were administered to rats subjected to sciatic nerve injury in order to shed light on the role of BDNF. The maintenance of synapses on axotomized motoneurons induced by treadmill running was partially dependent on TrkB activation. Treatment with the TrkB agonist at a low dose, but not at a high dose, prevented the decrease of excitatory glutamatergic synapses, and both doses increased the density of inhibitory synapses. TrkB inactivation counteracted only some of the positive effects exerted by exercise after nerve injury, such as maintenance of excitatory synapses surrounding motoneurons. Therefore, specific regimes of physical exercise are a better strategy to attenuate the alterations that motoneurons suffer after axotomy than pharmacological modulation of the TrkB pathway.

Key words: nerve injury, treadmill running, BDNF, TrkB, motoneurons, synaptic stripping

INTRODUCTION

Peripheral nerve injury results in loss of motor, sensory and autonomic functions in the denervated territory (Aldskogius and Molander, 1990; Navarro et al., 2007). Despite the fact that peripheral axons have the ability to regenerate, functional recovery is usually limited. This is attributable to insufficient numbers of regenerating axons, misdirection of regenerated axons leading to non-specific reinnervation of the target organs, and maladaptive changes of the neural central circuits (Navarro et al., 2007). The interruption of the contact between axons and their target organs is accompanied by disorganization of the central circuitry, in part due to the massive stripping of central synapses of axotomized motoneurons, being one of the most affected the proprioceptive afferent from muscle spindles (Alvarez et al., 2011).

A number of studies have shown that activity-dependent therapies improve nerve regeneration after peripheral nerve injury in animal models (Gordon and English, 2015; Park and Höke, 2014; Teodori et al., 2011; Udina et al., 2011a). Particularly, rats subjected to treadmill running exercise after sciatic nerve injury showed improved axonal regeneration (English et al., 2011), increased muscle reinnervation, and larger numbers of regenerated myelinated axons (Asensio-Pinilla et al., 2009).

Maladaptive plastic changes associated with peripheral nerve injury, such as hyperreflexia (Asensio-Pinilla et al., 2009; Vivó et al., 2008) and neuropathic pain (Cobianchi et al., 2010; Fu et al., 2004; Nam et al., 2001), can also be modulated by activity-dependent therapies. For instance, we found that an early treadmill running protocol reduces the excitability of sensory neurons (Modol et al., 2014) and attenuates the synaptic stripping and loss of perineuronal nets (PNN) that motoneurons suffer after axotomy (Arbat-Plana et al., 2015). Perineuronal nets create a barrier which limits neuronal plasticity, and their enzymatic degradation enhances functional recovery after spinal cord injury by promoting collateral sprouting and plastic changes in the central connections (García-Alías et al., 2009; Massey et al., 2006). In contrast, after peripheral nerve injury, preservation of PNN might limit the disorganization of the spinal circuits induced by the central disconnection of axotomized neurons.

Thus, the effects of exercise and other activity-dependent therapies are quite promising in animal models. Indeed, rehabilitation is one of the cornerstones of the treatment of neural injuries in humans. However, it is important to note that the intensity of the activity and the time of application after the injury are key factors that influence the beneficial effects (Tam and Gordon, 2003; Udina et al., 2011b). Different studies suggest that a high intensity

running protocol within a short time after axotomy is more beneficial than low intensity protocols (Arbat-Plana et al., 2015; Cobianchi et al., 2013), whereas delayed application of exercise after peripheral nerve injury is less effective (Brandt et al., 2015). In clinical practice, early application of activity-dependent therapies of moderate or high intensity can be difficult to achieve in traumatic patients. Therefore, a pharmacological approach that would mimic the effects of exercise on plastic changes observed after axotomy and that could also enhance axonal regeneration would allow early treatment of these injuries in patients.

It is well-known that exercise training modulates the concentration of different neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3) (Aszmann et al., 2004; Ying et al., 2005). Particularly, increased BDNF along with its specific receptor tropomyosin-related kinase (TrkB) induced by exercise has been proposed as underlying the significant enhancement of axonal elongation into peripheral nerve grafts (Sabatier et al., 2008) and the reduction of synaptic stripping on axotomized motoneurons (Krakowiak et al., 2015). However, another study proposes that physical exercise attenuates the development of neuropathic pain after nerve injury by reducing microglial expression of BDNF (Cobianchi et al., 2013, 2010; López-Álvarez et al., 2015). BDNF released by activated microglia induces down-regulation of the neuron-specific K^+-Cl^-cotransporter 2 (KCC2) (Coull et al., 2003; Ferrini and Koninck, 2013); this reduction is linked to the development of neuropathic pain and hypersensitivity after injury (Beggs et al., 2012; Granados-Soto et al., 2005).

Therefore, in this study we wanted to evaluate whether a TrkB agonist, 7-8-dihydroxyflavone (7,8-DHF), could mimic the positive effects of physical exercise in the spinal changes that motoneurons suffer after peripheral nerve injury and in the development of neuropathic pain. We also evaluated whether administration of a TrkB antagonist, ANA-12, would block the positive effects of exercise, with the aim of further investigating the role of the BDNF pathway in these spinal changes. 7,8-DHF is a small-molecule compound that crosses the blood-brain barrier and binds with high affinity and specificity to the BDNF receptor TrkB (Jang et al., 2010). Recent studies demonstrate that 7,8-DHF confers neuroprotective, cognitive-enhancing, and antidepressant effects in animal models (Andero et al., 2011; Blugeot et al., 2011; Devi and Ohno, 2012; Jang et al., 2010). In contrast, ANA-12 is a non-peptide antagonist of TrkB receptor (Cazorla et al., 2011). This molecule binds to the extracellular domain of TrkB, prevents BDNF-induced TrkB activation, and abolishes the biological effects of BDNF on TrkB-expressing cells but not those of NGF and NT-3 on

TrkA- and TrkC-expressing cells. Moreover, systemic administration of this compound inhibits TrkB function in the brain (Ambrogini et al., 2013; Cazorla et al., 2011).

MATERIAL AND METHODS

Experimental animals

Adult female Sprague Dawley rats (n = 45, 8 weeks old, 180–200 g) were used in this study. All rats were kept on standard laboratory food and water with a light-dark cycle of 12 h at room temperature of 22 ± 2°C. All experimental procedures were approved by the ethics committee of our institution and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). Before any surgical interventions, rats were anesthetized by intraperitoneal administration of ketamine (0.9 ml/kg; Imalgen 2000) and xylazine (0.5 ml/kg; Rompun 2%).

Retrograde labeling

Retrograde tracing was applied one week before intervention to identify motoneuron pools of tibialis anterior (TA) and gastrocnemius medialis (GM) muscles. Bilaterally, two retrotracers, Fluorogold (FG, Fluorochrome) and True Blue Chloride (TB, Setareh Biotech), were used to identify both motoneuron pools. A small incision to the skin was made to expose the muscle, and then two injections (2.5 µl/injection) were distributed within the muscle with a glass pipette using a Picospritzer (Arbat-Plana et al., 2015; Davalos et al., 2005; Geremia et al., 2007). The tip of the pipette was left for one minute inside the muscle to avoid reflux. The area of application was rinsed with saline to clean any remnants of the tracer and the skin wound was sutured.

Surgical procedure for nerve injury

Seven days later, rats were anesthetized again; the right sciatic nerve was exposed at the mid-thigh and sharply transected. The proximal and distal nerve stumps were rejoined with two epineural 10-0 sutures. The wound was closed and disinfected with iodine povidone, and the rats were allowed to recover in a warm environment under observation.

Experimental design

Rats were divided into groups according to the treatment received, as detailed in Table 1. For controls, one group of rats did not receive any treatment after sciatic nerve injury, a

second group received DMSO at 17% to evaluate potential toxicity of the vehicle solution, and a third control group was subjected to treadmill exercise from 3 days after surgery without receiving any pharmacological treatment. Regarding pharmacological modulation of TrkB, two subgroups of rats were treated with the agonist 7,8-DHF either twice a day at a high dose (10mg/kg) or once daily at a low dose (5mg/kg). The antagonist ANA-12 was given to two subgroups of rats, and one of them was also subjected to the treadmill running protocol from 3 days after surgery. Five rats per group were used for both immunohistochemical analyses and mechanical nociceptive testing, and the other 6 rats were used for western blot analyses (Fig.1).

Pharmacological treatments

The high-affinity TrkB receptor agonist 7,8-DHF (TCI-Europe) was injected intraperitoneally at a high and a low dose regime, according to previous works. In one group of rats 7,8-DHF was given twice a day at a high dose (10 mg/kg b.w. in 17% DMSO) (Jang et al., 2010), and in another group once daily at a low dose (5 mg/kg b.w. in 17% DMSO) (Agrawal et al., 2015; English et al., 2013). The TrkB receptor antagonist ANA-12 (Sigma-Aldrich) was administered intraperitoneally twice a day (0.5 mg/kg b.w. in 1% DMSO) (Cazorla et al., 2011). All pharmacological treatments started 3 days post-injury and were carried out for 2 weeks.

Treadmill running

Prior to surgery, rats subjected to training exercise were placed on a motor-driven rodent treadmill (Treadmill LE 8706, LETICA, Spain) for 60 min twice a week to get them used to the device. During these training sessions, shock grid intensity was set at 0.4 mA to provide a mild negative stimulus. The training protocol started 3 days after surgery and lasted until 2 weeks post-injury. Treadmill consisted of one session of treadmill running 5 days/week with increasing duration and progressively increased intensity; running started at a locomotion speed of 10 cm/s that was increased by 2 cm/s every 5 min, until a maximum speed of 30 cm/s for 60 min was reached (Cobianchi et al., 2013).

Mechanical nociceptive threshold measurement

Seven days before surgery all the rats were habituated to the experimental device, and then tested to obtain baseline nociceptive thresholds. The nociceptive behavior tests were

performed on both hind paws on different days after injury. The experimenter was blind to assignment of the rats to the different groups.

Sensitivity to mechanical stimulus was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed into individual transparent plastic cubicles with a wire-mesh floor. A mechanical stimulus was delivered to the medial plantar paw area, and then the pressure was slowly increased. The threshold was expressed as the force (in grams) at which rats withdrew the paw in response to the stimulus. A cutoff was set at 40 grams, when the stimulus lifted the paw without response. Threshold was calculated as the mean of three measurements, made with a minute's interval between each, and expressed as the percentage of the injured paw versus the intact contralateral paw. Algesimetry tests were performed during the morning, while treadmill sessions were carried out during the afternoon.

Immunohistochemical analysis of spinal changes

At the end of the two weeks' follow-up, rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in PBS. Lumbar spinal cord sections (L3–L6) were fixed in 4% paraformaldehyde for 4h and then cryoprotected in 30% sucrose and stored at 4°C until use. Samples were embedded in Tissue-Tek, serially cut in 20 μm thick transverse sections with a cryostat, and collected onto gelatin-coated glass slides. Sections were washed in PBS (0.1 M) initially and after every step. Sections were blocked for 1 h in normal bovine serum (10% FBS) and PBS with 0.2% Triton-X, followed by an incubation at 4°C overnight of primary antibodies (see Table 2). After washes, species-specific secondary antibodies conjugated to 488 Alexa Fluor (1:200, Invitrogen), 538 Alexa Fluor (1:500, Invitrogen), Cy3 (1:200 Millipore), or streptavidin 488 Alexa Fluor (1:200, Invitrogen) were applied, and samples were incubated at room temperature for 2 h. After final washes, sections were mounted and cover slipped with Fluoromount-G (SouthernBiotech). The samples processed for KCC2 were counterstained with DAPI and mounted with Mowiol (Sigma). Labeled motoneurons were localized and images captured with a scanning confocal microscope containing a z-stack(12) of all optical planes (LSM 700 Axio Observer, Carl Zeiss 40×/1,3 Oil DIC M27). To quantify the different immunolabelings, maximum intensity projections were applied to create an output image each of whose pixels contains the maximum value over all images in the stack at the particular pixel location.

Image analysis, processing, and regression analysis of all labeling quantification were performed by means of in-house software implemented in MatlabR2014b (The Mathworks Inc, Natick, MA, USA). Motoneurons were automatically selected by a Matlab script based on retrotracer intensity, and then manually verified. A constant threshold was used to segment and obtain the estimated average density for each labelling (in μm^2), in a perimeter of 5 μm surrounding the motoneuron soma (Arbat-Plana et al., 2015). For each rat, 10 to 15 motoneurons of each pool and each side were analyzed.

Non-consecutive sections of L4-L5 spinal cords of three different rats per group were randomly used for quantification of KCC2 immunoreactivity. Images of the sections were acquired with the confocal microscope at 20X magnification, and then analyzed using ImageJ software (NIH, USA). A fixed gray scale cutoff point set from the same image of control rats was used as threshold to subtract background. Then, the integrated density (InDen) of KCC2 labeling was measured.

Immunoblot

Rats were anesthetized and decapitated at 7 days post-axotomy for sample preparation (n=6 for each treatment). The L4–L5 spinal cord segment was removed and divided into quarters. The ipsilateral and contralateral ventral parts of L4–L5 cord segments were separately homogenized in RIPA modified buffer [50mM Tris-HCl pH 7.4, 1% Triton X-100, 0.5% DOCNa, 0.1% SDS, 150mM NaCl, 2mM EDTA] with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Roche). After clearance, protein concentration was measured with the BCA method assay (BCA Protein Assay kit, Pierce, Rockford, IL, USA). An equal amount of protein (40 μg/lane) was resolved in 7.5% SDS-PAGE and electrotransferred to PVDF membranes (Millipore) in transfer buffer [25mM trizma-base, 192mM glycine, 20% (v/v) methanol, pH 8.4]. Membranes were blocked with 6% non-fat dry milk in TBS plus 0.1% Tween 20 buffer for 1 h at room temperature and incubated overnight with the corresponding primary antibody diluted in blocking buffer: goat anti-TrkB and goat anti-TrkC (1:1000) (R&D Systems, Minneapolis, MN, USA), rabbit anti-phospho-AKT (S473)(D9E) and rabbit anti-pan-AKT (C67E7) (1:1000) (Cell Signaling Technology; Beverly, MA, USA), and mouse anti-β-Actin (Sigma-Aldrich). After several washes, membranes were incubated for 1 h with an appropriate secondary antibody conjugated with horseradish peroxidase (1:3000) anti-rabbit-HRP, anti-goat-HRP, or anti-mouse-HRP (Bio-Rad Laboratories, Berkeley, CA, USA). Proteins were visualized with the

enhanced chemiluminescence method (ECL Clarity kit, Bio-Rad Laboratories, Berkeley, CA, USA), and the images were captured and analyzed with Image Lab software (Bio-Rad Laboratories).

Statistical analysis

For quantitative variables, normality was assessed with the Shapiro-Wilk test (Royston, 1993). For variables with a normal distribution one-way ANOVA was used to test the significance of the difference between the lesion side and the contralateral side. For non-normal variables such analysis was performed with the Kruskall-Wallis test. A nested design ANOVA test was used in order to determine if the variability was due to the difference between the motoneurons or between rats in the experimental groups. Statistical analysis of nociceptive threshold was made with two-way ANOVA with group and time after injury as factors, followed by Bonferroni post hoc comparisons. Statistical significance for KCC2 immunofluorescence was calculated with two-way ANOVA (for multiple groups comparison) followed by Tukey post-hoc test when necessary. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A value of $p<0.05$ was considered significant. Data are presented as mean±SEM.

RESULTS

As we previously described (Arbat-Plana et al., 2015), axotomized motoneurons showed massive synaptic stripping of their central dendritic arbor, with a marked decrease in proximal glutamatergic synapses and an increase in inhibitory ones after nerve injury. There was also disorganization of their PNN, evidenced by a reduction of WFA immunolabeling.

We tested the higher dose of DMSO in control rats to rule out a toxic/beneficial effect of the vehicle. No significant differences were found between the non-injected group and the group injected with DMSO for any of the analyzed staining (data not shown). Thus, the DMSO group was used as a reference control.

Regarding the effects of the treadmill running protocol, we found that high intensity exercise reduced synaptic stripping in axotomized motoneurons, attenuating the loss of glutamatergic synapses and the increase of VGat synapses, and partially preventing the reduction of PNN around these motoneurons, (Figs. 2, 3). These findings corroborated the previously reported results (Arbat-Plana et al., 2015).

Synaptic stripping of injured motoneurons after TrkB modulation

Synaptophysin (Syn) immunolabeling, used as a general marker of synaptic coverage, showed a reduction of synaptic contacts after sciatic nerve injury. Both groups of rats treated with the TrkB agonist had significantly higher numbers of synaptic contacts on TA motoneurons compared to control injured rats ($p<0.001$), whereas this preservation was not significant in GM motoneurons (Figs. 2A, 3; Table 3). Preservation of Syn labeled contacts by 7,8-DHF was similar to that observed in the group of animals subjected to treadmill exercise.

In contrast, administration of ANA-12 further decreased the synaptic contacts around injured motoneurons, with this decrease significantly lower than in the control group ($p< 0.001$). Moreover, when animals submitted to exercise also received ANA-12, the positive effects of exercise on synaptic preservation were blocked, and similar values for synaptophysin immunoreactivity were observed in ANA-12 untrained and ANA-12 trained rats (Figs. 2A, 3; Table 3).

VGlut1 immunolabeling was used to label excitatory glutamatergic contacts mainly from proprioceptive afferents (REF ALVAREZ).We found that axotomized TA and GM motoneurons lost about 50% of these contacts compared to the contralateral side. The low dose of 7,8-DHF partially preserved VGlut1 density ($p<0.005$), although less than in the group subjected to exercise ($p<0.001$ vs control group). In contrast, rats treated with 10 mg/kg 7,8-DHF showed a more marked decrease of VGlut1 synapses in axotomized motoneurons of both TA and GM pools, significantly lower than in the control group ($p<0.001$). Like the high dose of 7,8-DHF, ANA-12 administration also induced a more marked decrease in VGlut1 synapses in injured motoneurons compared to nerve-injured controls. When rats treated with ANA-12 were subjected to treadmill exercise, the decrease in VGlut1 was less marked but still significantly lower than with treadmill running alone ($p<0.005$) (Fig. 2B, 3; Table 3).

VGat immunolabeling was used to label inhibitory gabaergic synapses. In contrast to Syn and VGlut1 immunoreactivity, VGat labeling surrounding axotomized motoneurons increased after injury compared to intact motoneurons. Treatment with 7,8-DHF produced a significantly higher increase in VGat contacts, more pronounced in the rats receiving the high dose than the low dose compared to untreated rats ($p<0.001$). In the group of rats subjected to treadmill exercise, VGat density surrounding axotomized motoneurons was close to contralateral values and significantly lower than in the 7,8-DHF-treated groups ($p<0.001$) (Figs. 2C, 3; Table 3). The noted increase in VGat inhibitory synapses after injury was less marked in ANA-12-treated rats compared to nerve-injured controls ($p< 0.001$). On the other hand, when ANA-12-treated rats were subjected to exercise, a lower increase in inhibitory

synapses was observed, with values similar to non-treated exercised animals (Figs. 2C, 3; Table 3).

It is worth noting that some of the treatments also affected the synaptic content of contralateral intact motoneurons. Indeed, the high dose of 7,8-DHF decreased Syn contact immunolabeling in these motoneurons, whereas VGlut1 immunoreactivity was lower in contralateral motoneourons of rats receiving both 10 mg/kg 7,8-DHF ($p<0.001$) and 5mg/kg 7,8-DHF ($p<0.001$) compared to rats of the control group (data not shown).

PNN around motoneurons after exercise and pharmacological modulation of trkB

PNN, immunolabeled against Wisteria floribunda, showed a reduced density around axotomized motoneurons that was partially prevented if rats were subjected to exercise. In contrast, administration of either low or high doses of 7,8-DHF did not affect the reduction of PNN observed after injury (Figs. 2D, 3; Table 3).

In the group of rats treated with ANA-12, we detected an increase in PNN immunoreactivity compared to untreated rats, although these differences reached statistical significance only for the GM motoneurons. Axotomized motoneurons of rats receiving ANA-12 and subjected to exercise showed a similarly significant increase in PNN staining compared to motoneurons of rats treated only with the trkB antagonist. This increase was similar to that observed in untreated rats subjected to treadmill exercise (Figs. 2D, 3; Table 3).

Glia reactivity around injured motoneurons

Astroglial reactivity, analyzed with GFAP immunolabeling around motoneurons, was significantly increased in the groups of rats treated with 7,8-DHF and with ANA-12 compared to the non-treated group. In the group of rats subjected to TR, astroglia reactivity was increased similarly in treated rats (Figs. 2E, 3; Table 3).

Iba1 immunoreactivity around axotomized motoneurons was used to evaluate microglial reactivity. Two weeks after nerve injury, there was a marked increase in microgliosis. The high dose of 7,8-DHF significantly increased microglial reactivity, whereas the low dose drastically reduced it, similar to what was observed in the exercised group (Fig. 2G, 3; Table 3). ANA-12 also reduced microglia reactivity ($p < 0.01$) around axotomized motoneurons compared to control injured rats (Fig. 2E, 3, Table 3), and did not affect the modulation of microglial reactivity induced by exercise (Fig. 2F, 3; Table 3).

Changes in KCC2 expression in the spinal cord

A significant decrease in KCC2 immunoreactivity was observed after peripheral nerve injury at both ventral and dorsal horns of the injury side compared to intact rats (Fig. 4A, naïve vs. DMSO groups). Neither 7,8-DHF nor ANA-12 treatment significantly changed this effect (images not shown). In contrast, treadmill exercise prevented the decrease in KCC2 immunoreactivity, this effect being more evident in the group with combined ANA-12 treatment. Statistical comparisons of KCC2 immunoreactivity indicated significant decrease of all injured groups, except groups performing treadmill running with respect to naïve rats in the injured side dorsal and ventral horns and also in the contralateral dorsal horn of the spinal cord (Fig. 4B, $p<0.01$). On the other hand, after injury, KCC2 immunoreactivity was significantly higher in the injured dorsal horn of the ANA-12+TR group compared to the other injured groups not subjected to treadmill exercise ($p<0.001$, ANA-12+TR group vs. DMSO, ANA-12 and both 7,8-DHF groups).

Effect of treadmill running and drug administration on mechanical nociception

Sciatic nerve injury induced a decrease of the withdrawal threshold in the injured paw with a mechanical stimulus, rapidly lowering to 58% and 38% of contralateral values at 3 and 14 days post-injury, respectively, in the control groups (Fig. 5), reflecting a state of mechanical allodynia. Similar to what was previously demonstrated (Cobianchi et al., 2013; López-Álvarez et al., 2015), the treadmill running protocol used produced a significant increase in the withdrawal threshold of the injured paw compared with the control group, both at 7 (70% of contralateral; $p<0.001$) and 14 days (71%; $p<0.001$). In contrast, administration of the low dose of 7,8-DHF resulted in a further threshold decrease at 7 days compared to exercised rats, whereas the high dose of 7,8-DHF induced no changes compared with the DMSO group. ANA-12 administration did not change the withdrawal threshold compared to the DMSO group; however, when combined with treadmill exercise, it did not change the hypoalgesic effect (67% at 7 days, $p<0.01$ in DMSO vs. ANA-12+TR groups; 82% at 14 days, $p<0.001$ in DMSO vs. ANA-12+TR groups). The mechanical withdrawal threshold of the treadmill group was significantly higher at 7 and 14 days compared with the drug treatment groups (Fig 5).

Effect of 7,8-DHF and treadmill running on protein levels of TrkB, TrkC and AKT

Protein levels of TrkB in the spinal cord were not affected by the sciatic nerve injury. TrkB expression tended to increase in the injured side of rats subjected to exercise, although

the difference was not significant. Administration of 7,8-DHF did not affect TrkB expression compared to untreated rats (Fig 6A,B). In addition, TrkC protein levels were not increased after axotomy, either after 7,8-DHF or treadmill running treatments. Finally, levels of AKT, a downstream signaling pathway of tyrosine-kinase receptors, were similar in 7,8-DHF and exercised rats, and higher than in untreated rats, although differences were not significant (Fig. 6).

DISCUSSION

The results of this study show that a high intensity protocol of treadmill running applied for 2 weeks is able to attenuate synaptic stripping and PNN loss around the injured motoneurons after sciatic nerve lesion in rats, corroborating the results obtained in previous studies (Arbat-Plana et al., 2015; Cobianchi et al., 2013). Since physical exercise modulates the expression of BDNF and NT-3 in the spinal cord, with specific patterns of activation for each neurotrophin (Ding et al., 2011; Krakowiak et al., 2015; Skup et al., 2000), in this study we evaluated whether systemic administration of a TrkB analog, 7,8-DHF, could mimic the ability of treadmill exercise to reduce synaptic stripping of motoneurons and neuropathic pain after axotomy. To further clarify the role of TrkB activation, we also evaluated whether an antagonist of TrkB could block the positive effects induced by treadmill exercise. Since it has been previously reported that physical exercise has different effects on males and females in experimental models (English et al., 2011), in this study we specifically evaluated female rats, as we had previously characterized the effects of TR in this gender (Arbat-Plana et al., 2015). As in the prior study, we also analysed two motoneuron pools, corresponding to the extensor GM muscle and the flexor TA muscle, both innervated by the sciatic nerve. To differentiate the motoneuron pools, we injected two different retrotracers into these muscles and checked the motoneuron pool position in the spinal cord (Köbbert and Thanos, 2000). In the previous study, we found that the main changes surrounding motoneurons occurred at 2 weeks post-injury (Arbat-Plana et al., 2015). For this reason, in the present study all changes were considered at this time point after injury, at which axons are regenerating after a nerve cut and suture but the target muscles still remain denervated (Valero-Cabré et al., 2004).

In a previous study, administration of the TrkB agonist 7,8-DHF (10 mg/kg once a day) mimicked the effects that physical exercise and environmental exploration have on synaptogenesis in the rat dentate gyrus (Ambrogini et al., 2013). Another study showed that this agonist, administered at a lower dose (5 mg/kg once a day), promoted axonal regeneration in transected nerves (English et al., 2013). Therefore, we administered 7,8-DHF at both doses

to evaluate its effects on the plastic changes induced by peripheral nerve injury in the spinal cord. After nerve injury, there is an important loss of excitatory synapses and an increase in inhibitory synapses in motoneurons, which is partially prevented when animals are subjected to physical exercise, in the form of treadmill running (Arbat-Plana et al., 2015). A low dose of 7,8-DHF also favored the maintenance of synapses in axotomized motoneurons, whereas the antagonist of TrkB ANA-12 blocked the positive effects of exercise on synaptic preservation, thus indicating that activation of TrkB is important for the maintenance of synapses in spinal motoneurons. However, a higher dose of 10 mg/Kg of 7,8-DHF caused a further loss of excitatory synapses and a significant increase in inhibitory synapses. This finding suggests that synaptic maintenance induced by TrkB activation is dose-dependent, and over-activation can promote deleterious effects on synaptic content, even affecting intact motoneurons.

Although a low dose of TrkB agonist partially preserved VGlut1 loss in injured motoneurons, both high and low doses of 7,8-DHF reduced VGlut1 content of intact motoneurons. These drugs also increased VGat synapses in injured motoneurons, whereas ANA-12 blocked the positive effects of exercise on VGlut1 synapses but not VGat ones. Therefore, BDNF seems to be important in the maintenance of both inhibitory and excitatory synapses, with its effects on these excitatory synapses more sensitive to the amount of TrkB activation. In contrast, exercise mainly favors preservation of excitatory synapses and attenuates the increase in the inhibitory synapses induced by the injury. Therefore, exercise probably induces a more selective activation of TrkB than a systemic TrkB agonist administered at a high dose, along with an increase of other neurotrophic factors, like NT-3, and their receptors (Gómez-Pinilla et al., 2001; Krakowiak et al., 2015; Skup et al., 2000). In fact, in abducens oculomotor neurons, prevention of synaptic stripping was more effective when both NT-3 and BDNF were applied together than separately, and these neurotrophins had complementary, but not compensatory effects, on distinct afferents (Davis-López de Carrizosa et al., 2009).

Thus, to further elucidate the potential role of BDNF and NT-3 in the effects of exercise, and the reasons why a TrkB agonist does not mimic these effects, we evaluated the expression of their receptors, TrkB and TrkC, respectively, in female rats subjected to exercise or treated with 7,8-DHF. Levels of TrkB receptor tended to increase in the spinal cord of exercised rats, whereas administration of the TrkB agonist did not affect its levels. In contrast, levels of TrkC receptor were similar in the two groups. To corroborate that the TrkB pathway was being activated, we evaluated the downstream signaling AKT, which was

similarly expressed in the two groups. We did not find significant diferences in TrkB or AKT proteins after 7,-8-DHF administration compared to control injured rats, likely attributable to an overactivation of TrKB. It has been previously reported that prolonged BDNF treatment reduces the response of TrkB (Ascaño et al., 2009; Carter et al., 1995; Chen et al., 1995). Moreover, the expression of these three proteins was analyzed in the whole ventral spinal cord, and specific changes in some of the cell populations could be masked. Although the major source of BDNF appears to be neuronal (Rauskolb et al., 2010), it can also be produced by astrocytes and microglia (Dougherty et al., 2000; Parpura et al., 2010). In fact, English et al. (2013) showed that the effects of exercise are mainly mediated by activation of neuronal TrkB, whereas treadmill exercise seems to reduce activation of microglial TrkB (Cobianchi et al., 2010). Thus, whereas systemic administration of a TrkB analog would activate this receptor in all cell types, exercise could be acting more specifically in some cell populations, increasing activation of TrkB in neurons while reducing it in microglia.

It is known that microglia express activated TrkB, and released BDNF increases their proliferation (Spencer-Segal et al., 2011). The secretion of BDNF by microglia has been linked to the inversion of inhibitory GABAergic currents in neurons, triggered by KCC2 down-regulation (Coull et al., 2003; Ferrini and Koninck, 2013). GABAergic interneurons are particularly sensitive to altered BDNF signaling. TrkB activation mediated by activation of BDNF suppresses Cl^- dependent fast GABAergic inhibition since it down-regulates KCC2 (Rivera et al., 2001), inverting the anion flux upon GABA receptor activation from inhibitory to excitatory (Beggs et al., 2012; Price et al., 2005). After peripheral nerve injury there is an important down-regulation of both ipsilateral and contralateral KCC2 protein and its phosphorylated active form in dorsal horn neurons, which is associated with the development of neuropathic pain (Modol et al., 2014). We recently found that treadmill running significantly reduced microglia reactivity and BDNF expression in microglia after sciatic nerve injury, also rescuing KCC2 levels (López-Álvarez et al., 2015). Thus, exercise can counteract some of the mechanisms related to central disinhibition. Here we observed that treatment with the TrkB analog caused a trend toward further reduction of KCC2 levels after peripheral nerve injury, whereas administration of the antagonist ANA-12 in exercised rats increased these levels, suggesting that pharmacological TrkB modulation may partially affect KCC2. In contrast, administration of ANA-12 by itself is not able to mimic the analgesia induced by exercise, indicating that treadmill running may be acting on excitability of sensory neurons through mechanisms other than TrkB modulation. In fact, the reduction in microglia reactivity induced by treadmill running may be unrelated to the effects of exercise on

neuropathic pain, since this reactivity is not specifically affected by the different treatments, whereas the modulation of neuropathic pain is clearly dependent on TrkB activation. Low doses of the TrkB analog increased the hyperalgesia induced by peripheral nerve injury that was also accompanied by increased microglia reactivity in the dorsal horn. In contrast, pharmacological blockade of TrkB activity did not reproduce but may in fact have facilitated the hypoalgesia induced by TR. In fact, treadmill running significantly reduced hyperalgesia after axotomy, and this effect was potentiated by administration of the TrkB antagonist, which did not induce an increase in microglia reactivity after injury.

When focusing on PNN, we found that 7,8-DHF had no effects, whereas ANA-12 administration, both in exercised and non-exercised rats, significantly increased PNN density around axotomized motoneurons compared to control untrained female rats. Therefore, maintenance of PNN by exercise can be mediated by blockade of TrkB activation, probably on glial cells. In fact, during development, blockade of TrkB-mediated signaling by chondroitin sulfate proteoglycans, the main component of PNN, leads to closure of the critical period (Kanato et al., 2009; Kurihara and Yamashita, 2012) and thus restriction of neural plasticity. Although it has long been assumed that exercise increases neural plasticity, the maintenance of spinal PNN induced by physical activity, by limiting plasticity at this level, could reduce the disorganization of the spinal circuitry observed after injury and thus preserve functionality.

Despite the fact that some of the effects of exercise on central circuitry and plasticity after peripheral nerve injury are mediated through TrkB activation, pharmacological activation of TrkB receptor is not able to mimic all the positive effects of exercise and may even have deleterious effects on synaptic maintenance in motoneurons and facilitate the development of neuropathic pain. Therefore, the complex pattern of activation induced by specific regimes of physical exercise seems a better strategy to modulate the maladaptative plastic changes induced by nerve injury than systemic modulation of the BDNF pathway.

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FIGURE LEGENDS

Table 1. Experimental design. Groups and treatments applied. Animals were divided into 4 main experimental groups. In all of them except the naïve group the sciatic nerve was cut and repaired by direct suture. Control groups: vehicle (DMSO 17% twice a day) was administered in a group of rats; another group was untreated, with half of the rats submitted to treadmill running and the other half left untrained. TrkB agonist administration: two subgroups with different doses of 7,8-DHF treatments were evaluated. TrkB antagonist administration: the group of rats treated with ANA-12 was divided into two subgroups: one submitted to treadmill running and the other one untrained. Naïve group: non-injured rats without any treatment.

Experimental groups	**Pharmacological treatment**	**Exercise protocol**	
		Immunohistochemistry, nociceptive tests	**Immunoblotting**
Control (right sciatic nerve injury)	DMSO 17% (2/day)	No exercise (n=4)	-
	None	Treadmill (n=5)	Treadmill (n=6)
	None	No exercise (n=5)	No exercise (n=6)
TrkB activation (right sciatic nerve injury)	7,8-DHF (5 mg/kg 1/day)	No exercise (n=5)	No exercise (n=6)
	7,8-DHF (10 mg/kg 2/day)	No exercise (n=5)	-
TrkB inactivation (right sciatic nerve injury)	ANA-12 (0.5 mg/kg 2/day)	Treadmill (n=5)	-
		No exercise (n=5)	-
Naïve (no nerve injury)	None	No exercise (n=5)	No exercise (n=6)

Table 2. Antibodies used in the study.

Antigen	**Immunogen**	**Host**	**Working**	**Supplier**

			dilution	
VGlut1	Synthetic peptid from rat VGlutI	Guinea pig polyclonal	1:300	Millipore Ref AB5905
Vgat	Cytoplastic domain of vesicular GABA transporter	Guinea pig polyclonal	1:200	Synaptic systems
GFAP	Purified GFAP from porcine spinal cord	Mouse	1:1000	Millipore Ref AG230
Iba1	C-terminus of Iba-1 synthetic peptide	Rabbit polyclonal	1:500	Wako
Synaptophysin (Syn)	C-terminus of human synaptophysin	Rabbit polyclonal	1:200 and Biotin amplification	**Covance**
Perineuronal nets (PNN)	Lectin from Wisteria floribunda, Biotin conjugated	-	1:200	Sigma
KCC2	N-terminal His-tag fusion protein	Rabbit polyclonal	1:500	Millipore Ref 07-432

Table 3. Integrated density of immunolabeling of the different markers evaluated around axotomized motoneurons of the tibialis anterior (TA) and gastrocnemius medialis (GM) motor nuclei.

		Syn	**VGlut1**	**VGat**	**WFA**	**GFAP**	**Iba1**
DMSO 17%	TA	77±2	41±4	67±1	50±2	4±1	82 ± 18
	GM	88±2	39±2	103±7	56±4	3±2	80 ± 10
TR	TA	97±5	57±2	49±2	77±3	262±4	6±3
	GM	104±5	62±2	85±2	83±4	260±2	7±2

7,8-DHF high	TA	88±4	26±2	104±5	46±1	267±14	153±12
	GM	85±3	27±3	112±6	52±2	260±9	148±13
7,8-DHF low	TA	87±4	45±3	78±5	48±4	263±30	8±2
	GM	96±4	56±2	109±6	56±3	261±23	7±1
ANA-12	TA	67±5	28±4	55±4	52±3	254±9	5±3
	GM	80±3	29±2	96±3	78±6	258±8	4±2
ANA-12+TR	TA	65±3	40±2	46±3	61±4	272±8	4±2
	GM	68±1	46±4	90±6	80±6	269±9	5±2

Fig. 1. Diagram of the procedures performed in the study.

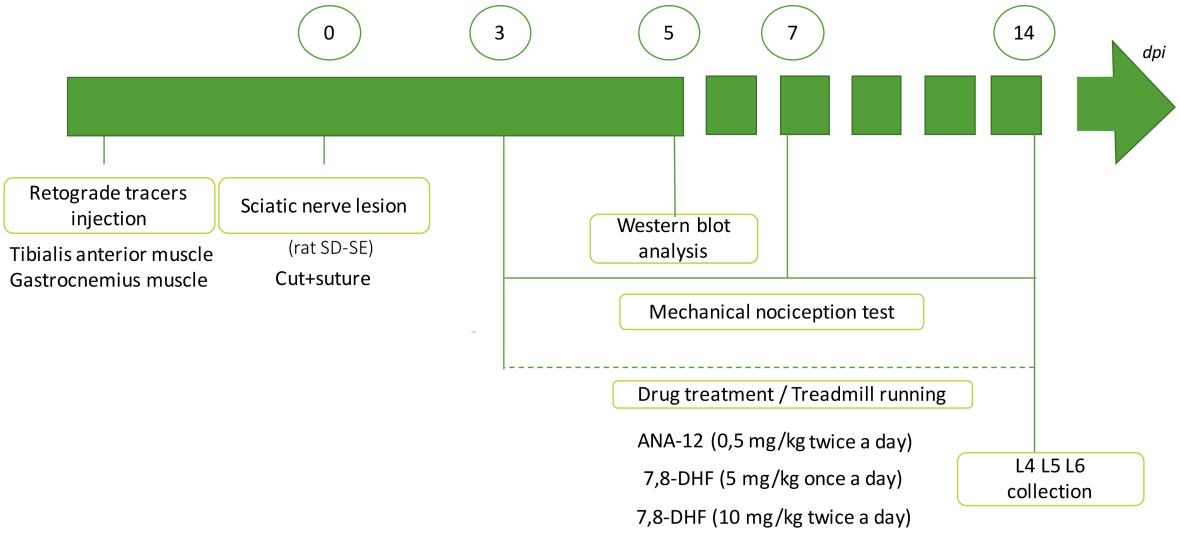


Fig. 2. Quantitative analysis of synaptic strippingVGlut1 and Vgat contacts,, PNN, and glia immunoreactivity on motoneurons after axotomy in the different experimental groups. Synaptophysin (A) a general marker for synapses, Vglut1 (B) vesicular glutamat transporter type 1, Vgat (C) vesicular GABA transporter, , WFA (D), a general marker for PNN, GFAP (E) to label astrocytes, and Iba1 (F) to label microglia, were evaluated in in back-labeled motoneurons of tibialis anterior (TA, gray bars) and gastrocnemius medialis (GM, white bars) muscles from injured rats either treated with DMSO 17%, treated with a

low and a high dose of 7,8-DHF, subjected to TR, treated with ANA-12, or treated with ANA-12 + TR. Horizontal dotted lines indicate the average of motoneuron labeling in the non-injured side of both TA and GM motoneurons. Data are expressed in absolute values as mean ± SEM, *p<0.05, **p<0.01.

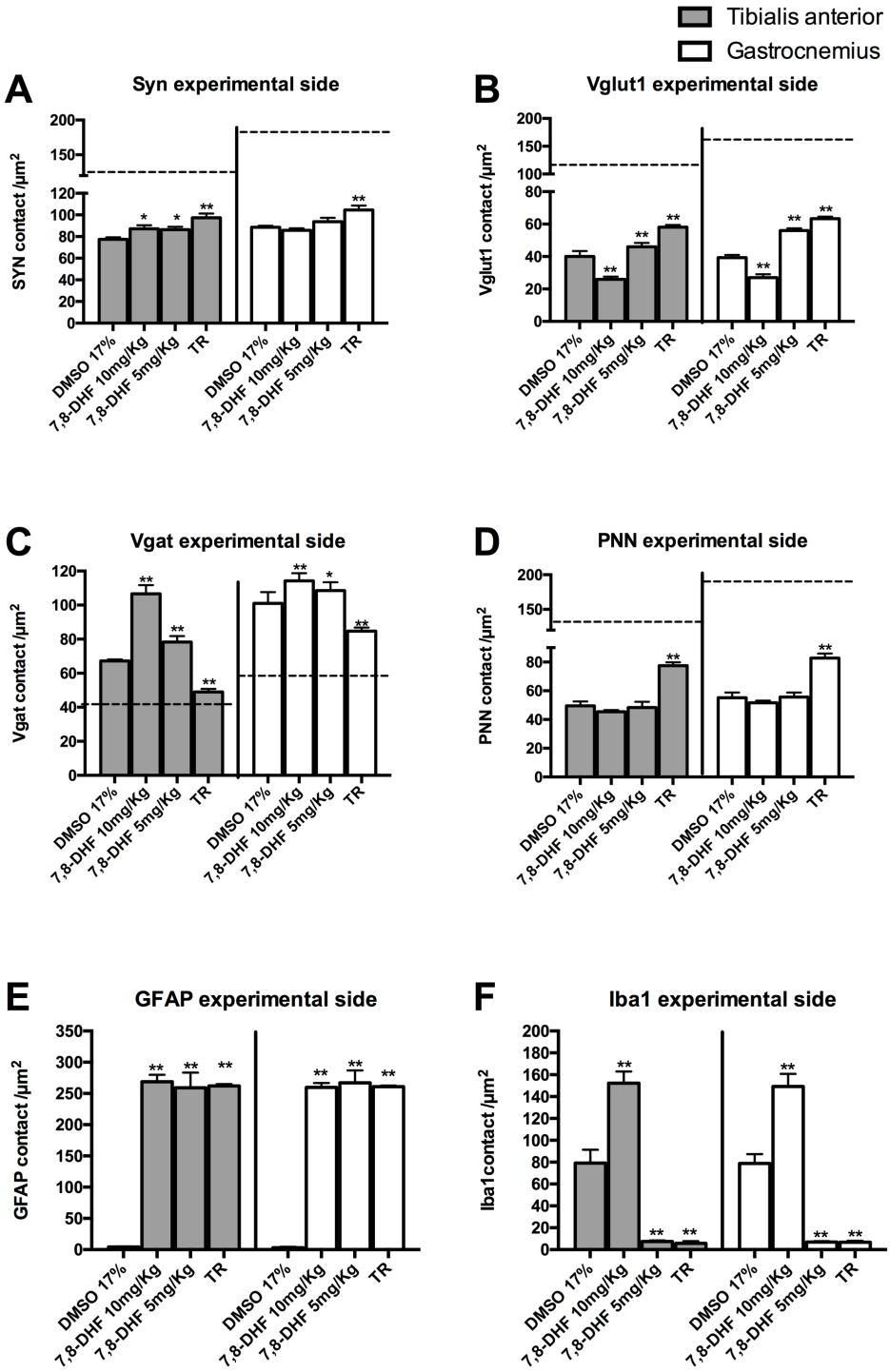


Fig. 3. Representative confocal images of immunostainings **to evaluate synaptic stripping, Vglut1 and Vgat contacts, PNN, astroglia and microglia reactivity around axotomized motoneurons of rats subjected to different treatments after sciatic nerve injury.** All immunostainings were assessed in confocal images of spinal cord regions containing back-labeled motoneurons (blue) of TA and GM muscles after sciatic nerve section and suture repair in the different groups of rats.

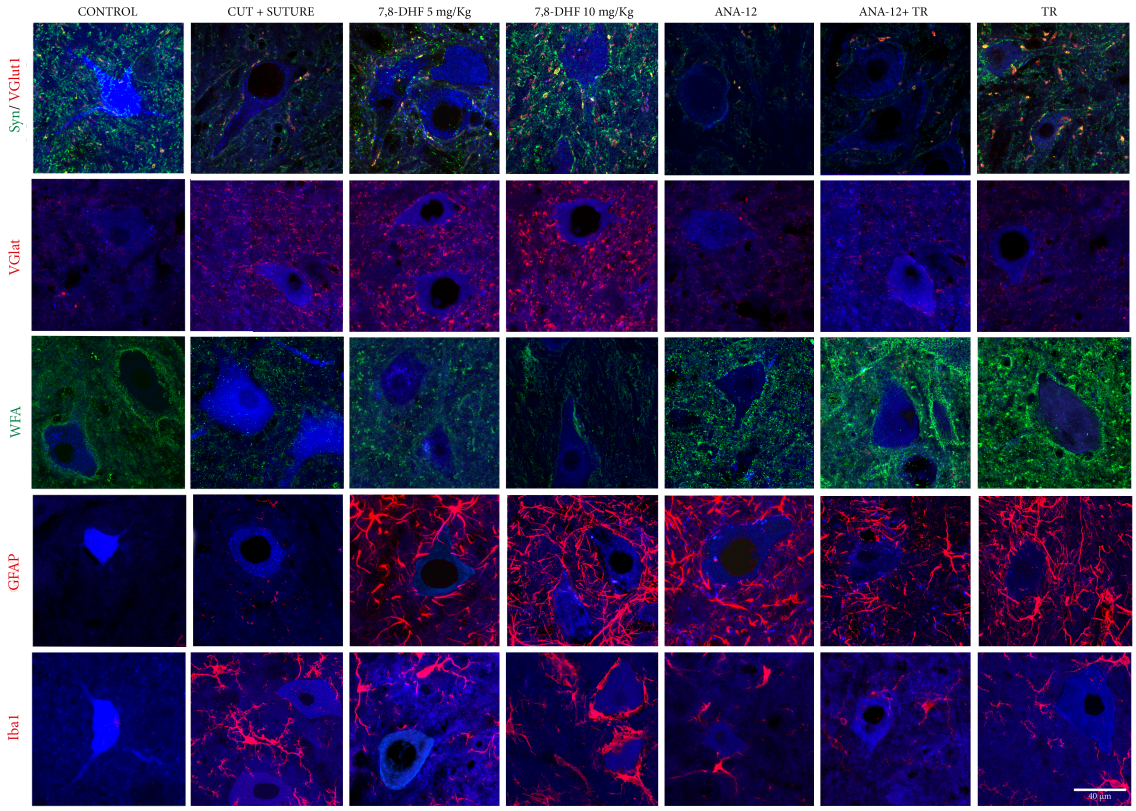
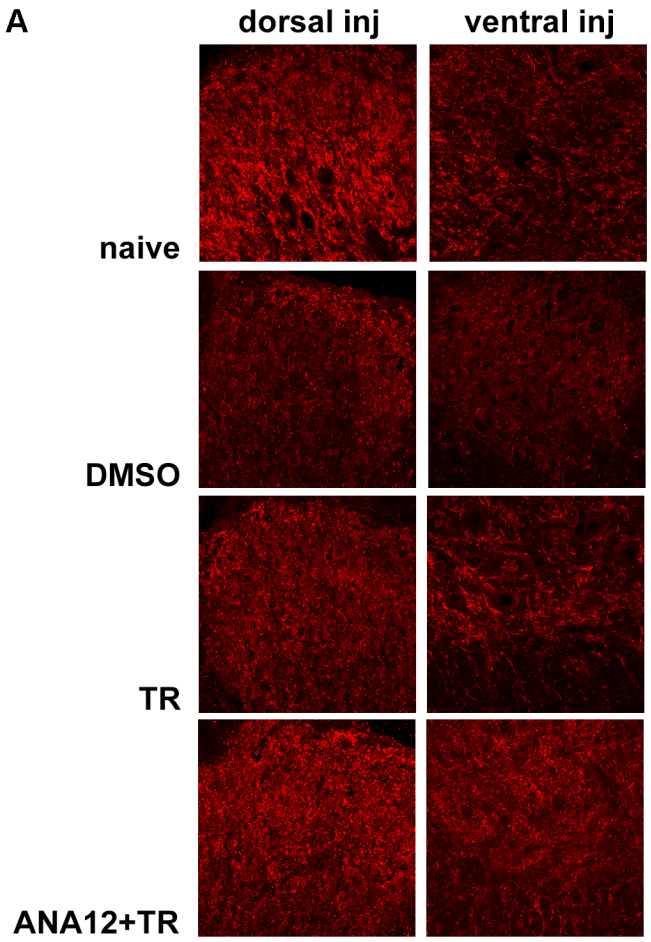
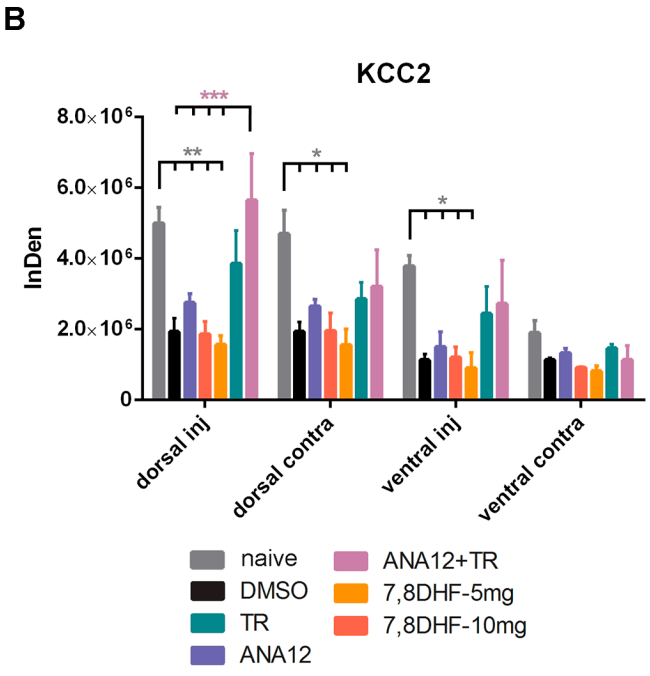


Fig. 4. Changes in KCC2 expression at dorsal and ventral horns of the spinal cord. A. Representative images of immunostaining against KCC2 in dorsal and ventral horns of spinal cords. Peripheral nerve injury induced an important reduction in the immunostaining against KCC2 in both spinal horns of the injured side (DMSO group). This reduction was prevented in rats performing exercise (TR and ANA-12+TR groups). In contrast, drug treatments alone did not change KCC2 immunoreactivity after injury (images not shown). **B**. Quantification of KCC2 immunoreactivity in L4-L5 spinal cords of the different experimental groups. Statistically significant differences were found between the naïve and other groups (gray asterisks) and between ANA-12+TR and other groups (violet asterisks). Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.


A
dorsal inj
ventral inj
naive
DMSO
TR
ANA12+TR


B
KCC2
InDen
8.0×10⁶
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4.0×10⁶
2.0×10⁶
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*
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dorsal inj
dorsal contra
ventral inj
ventral contra
naive
DMSO
TR
ANA12
ANA12+TR
7,8DHF-5mg
7,8DHF-10mg

Fig. 5. Mechanical algesimetry test results of rats subjected to different treatments. Values are expressed as the % ratio between the threshold force ipsilateral versus contralateral to the injured paw at different days post-injury (dpi). Significant statistical comparisons are represented for DMSO and TR groups versus drug treatment groups after injury. Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

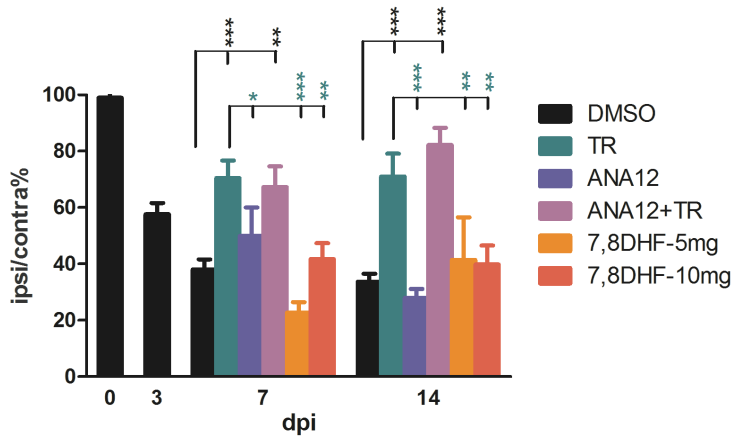

Fig 6. Western blot analysis of the tyrosine-kinase receptors. (A) Immunoblot at dpi of the contralateral (c) and injured (i) sides of the rat spinal cord ventral horn of naïve (N), axotomized (C+S), treated with 7,8-DHF (7,8-DHF), and exercised (TR) groups (B-D). All protein levels were normalized with β-actin and analyzed with Image Lab software (Bio-Rad Laboratories, Inc, Berkeley, CA, USA). Data are expressed as mean ± SEM, *p<0.05, **p<0.01.